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Phosphorylation of CREB Ser142 Regulates Light-Induced Phase Shifts of the Circadian Clock

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Summary

Biological rhythms are driven in mammals by a central circadian clock located in the suprachiasmatic nucleus (SCN). Light-induced phase shifting of this clock is correlated with phosphorylation of CREB at Ser133 in the SCN. Here, we characterize phosphorylation of CREB at Ser142 and describe its contribution to the entrainment of the clock. In the SCN, light and glutamate strongly induce CREB Ser142 phosphorylation. To determine the physiological relevance of phosphorylation at Ser142, we generated a mouse mutant, CREB^{S142A}, lacking this phosphorylation site. Light-induced phase shifts of locomotion and expression of c-Fos and *mPer1* in the SCN are significantly attenuated in CREB^{S142A} mutants. Our findings provide genetic evidence that CREB Ser142 phosphorylation is involved in the entrainment of the mammalian clock and reveal a novel phosphorylation-dependent regulation of CREB activity.

Introduction

Most physiological and behavioral processes follow circadian rhythms (Aschoff, 1981) that are driven by a central autonomous clock located in the suprachiasmatic nucleus (SCN) (Reppert and Weaver, 2001; Wager-Smith and Kay, 2000). The molecular basis of circadian rhythm generation is formed by interlocking transcriptional and translational feedback loops. Under natural conditions, circadian rhythms are synchronized to the 24 hr cycle by external time cues (Zeitgeber), the most important being the day-night cycle. Indeed, light pulses applied during early or late subjective night result in phase delays or advances, respectively, while light pulses administered during the subjective day have no influence on the circadian locomotor activity (Pittendrigh and Daan, 1974). The molecular mechanism by which light entrains the circadian clock is believed to involve activation of NMDA receptors in the SCN by glutamate with subsequent influx of Ca^{2+} (Ding et al., 1997; Schurov et al., 1999). The excitatory glutamatergic input is coupled to several signal transduction pathways leading to chromatin modifications (Crosio et al., 2000), modification of clock proteins (Myers et al., 1996), and activation of immediate-early genes, such as c-*fos* (Kornhauser et al., 1990) and the clock genes *Per1* and *Per2* (Albrecht et al., 1997; Zylka et al., 1998). Induction of gene expression by light is correlated with shifts of the circadian clock (Shigeyoshi et al., 1997). Interestingly, the phase delays and advances in the expression of clock genes perfectly correspond to the delay in the behavioral rhythm in rodents (Schwartz and Zimmerman, 1990; von Gall et al., 1998).

In several studies, a role of the cyclic AMP responsive element binding protein (CREB) has been demonstrated in the resetting of the circadian clock, since light-induced phase shifts are accompanied by rapid phosphorylation of CREB at Ser133 (Ding et al., 1997; Ginty et al., 1993) and CRE-dependent transcription (Obrietan et al., 1999) in the SCN. Moreover, cAMP has been shown to reset the mammalian clock in vitro (Prosser and Gillette, 1989). However, genetic proof that CREB participates in the entrainment mechanism of the mammalian clock is still lacking.

CREB function is regulated by phosphorylation at Ser133 (Mayr and Montminy, 2001) and possibly by additional phosphorylation events (Fiol et al., 1994). In vitro, calcium/calmodulin-dependent kinase II α (CaMKIIα) is able to phosphorylate CREB at Ser133 and Ser142 (Sun et al., 1994). Using transient transfections, a possible role for Ser142 phosphorylation was described (Sun and Maurer, 1995). Whereas the phosphorylation of Ser133 has been demonstrated to be essential for CREB activity (Yamamoto et al., 1988), phosphorylation at Ser142 in vivo and its functional significance remains an open question (Mayr et al., 2001).

Here, we show that the phosphorylation of CREB at Ser142 occurs in vivo by using an antibody specific for the phosphorylated form of Ser142. Moreover, using a mouse mutant lacking this phosphorylation site (CREB^{S142A}), we provide evidence that this phosphorylation participates in the synchronization mechanism of the circadian clock.

Results

CREB Phosphorylation at Ser142 Occurs In Vivo

To determine whether CREB is phosphorylated at Ser142 in vivo and define the role of this phosphorylation, we generated an antibody specific to the phosphor-

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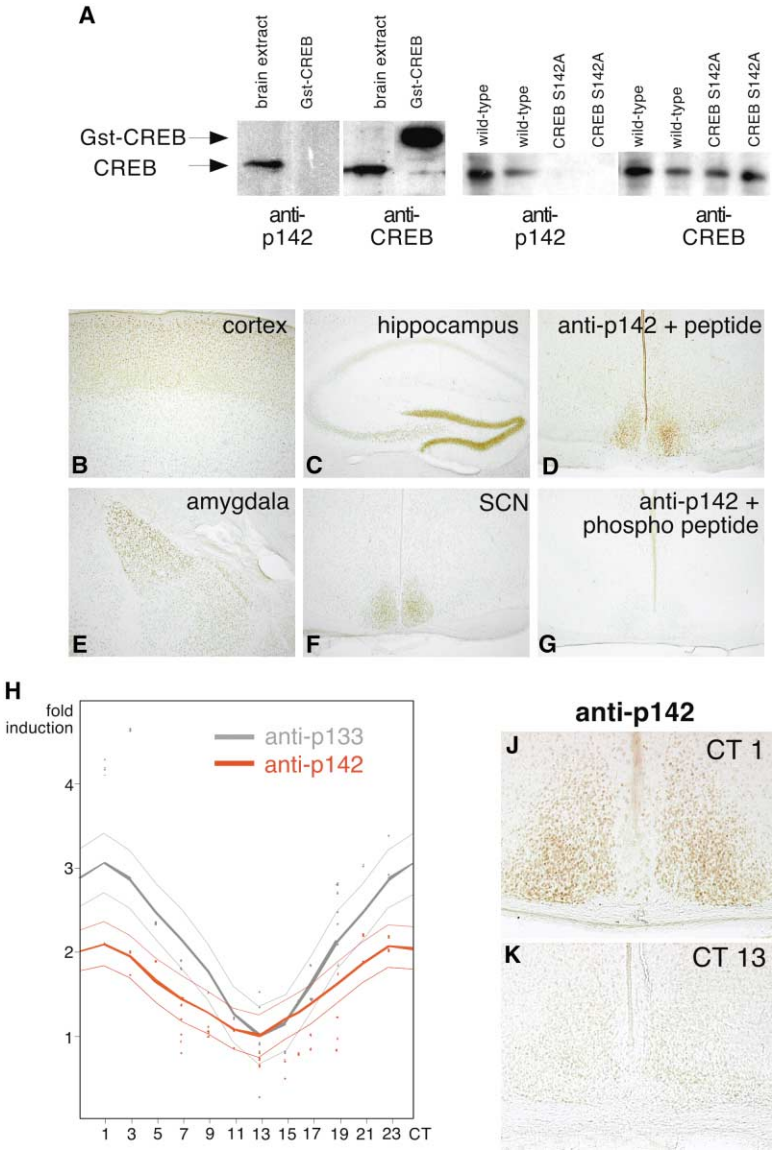


Figure 1. CREB Ser142 Phosphorylation in the Brain

(A) Western blot analysis of the anti-p142 antibody. Comparison of bacterial overexpressed GST-CREB and nuclear brain extracts of wild-type and CREB^{S142A} hippocampus.

(B–G) Detection of CREB Ser142 phosphorylation in the brain using the anti-p142 antibody. Animals were sacrificed around circadian time (CT) 23.

(H) Quantification of CREB phosphorylation at Ser142 and Ser133 in the SCN during the circadian cycle. C57Bl/6 mice were transferred to constant darkness for 5 days and sacrificed every 2 hr under dim red light (n = 4–8 per time point). 95% coincidence intervals are indicated.

(J and K) Representative anti-p142 stainings are given at CT 1 and CT 13.

ylated form of CREB Ser142 (anti-p142). The specificity of the anti-p142 antibody was tested by comparing its recognition of bacterially overexpressed CREB (GST-CREB) and CREB from nuclear brain extract. The anti-p142 antibody detects only a single band corresponding to the phosphorylated CREB protein in wild-type nuclear brain extracts and does not cross-react with the unphosphorylated bacterially expressed GST-CREB (Figure 1A). Furthermore, the signal is only detected in nuclear extracts from wild-type mice, but not from CREB^{S142A} mutant mice, lacking this phosphorylation site (see below). Together, these results show that this antibody specifically recognizes the phosphorylated form of CREB Ser142.

In the mouse brain, strong staining by anti-p142 was specifically detected in several regions, including the SCN, the amygdala, the cortex, and the hippocampus (Figures 1B, 1C, 1E, and 1F). In contrast, no staining for CREB Ser142 was found in the striatum or the cerebellum. Recognition of the anti-p142 antibody is disrupted by competition with the phosphorylated peptide, but not with the unphosphorylated peptide, further confirming specificity of the antibody (Figures 1D and 1G).

Phosphorylation of CREB Ser142 in the SCN

Several studies suggest a role of CREB in the mammalian central circadian clock, since phosphorylation at Ser133 is oscillating in cells of the SCN during the circadian cycle and is induced upon nighttime light treatments (Ginty et al., 1993; Obrietan et al., 1999). This role of CREB is supported by the circadian activity of the *Drosophila* CREB/CREM homolog dCREB2 (Belvin et al., 1999). The fact that phosphorylation of CREB at Ser142 is detectable in the SCN prompted us to analyze whether this phosphorylation also follows a circadian oscillation. We therefore monitored the phosphorylation of CREB at Ser142 in the mouse SCN at different times, with circadian time (CT) 12 being defined by the onset of darkness had the animals remained in a 12 hr light:12 hr dark cycle. Phosphorylation of CREB Ser142 was low in animals sacrificed at CT 9–15 (Figure 1H). However, with the beginning of the active phase, phosphorylation

of Ser142 increased significantly and reached a maximum in the early subjective day at CT 1 (Figures 1J and 1K). Phosphorylation of CREB Ser142 in the SCN thus parallels the circadian oscillation of Ser133 phosphorylation. The phase of CREB Ser142 and Ser133 phosphorylation precedes the one of the CRE-dependent expression in transgenic mice (Obrietan et al., 1999). This observation suggests that both CREB phosphorylations at Ser142 and Ser133 may regulate the activity of CREB and participate in circadian rhythm generation. Although CREB Ser142 phosphorylation can be detected in other brain areas, such as the hippocampus, amygdala, and cortex, circadian oscillation of CREB Ser142 phosphorylation was restricted to the SCN (data not shown).

We then investigated whether phosphorylation of CREB at Ser142 occurs in response to the stimuli (e.g., light and glutamate) that are critical for the synchronization of the circadian clock to the external day-night cycle. We first tested the effect of light. Entrained animals were transferred to constant darkness for 48 hr and then received a 15 min light pulse at different circadian times. We looked for induced phosphorylation of CREB Ser142 upon photic stimulation in neurons of the basal SCN and compared it to the phosphorylation of CREB Ser133. Both CREB Ser142 and Ser133 were phosphorylated following a light stimulus applied during early subjective night (CT 14) in neurons of the basal SCN (Figure 2A). Photic stimulation during late subjective day at CT 10, which has no effect on the circadian clock, did not elicit phosphorylation at Ser142 and Ser133 (Figure 2A). Light is, therefore, an efficient stimulus able to trigger Ser142 phosphorylation in vivo. Since the entrainment of the central circadian clock by light depends on direct excitatory glutamatergic projections of the retinal ganglion cells to the basal neurons of the SCN (Ding et al., 1997), we further analyzed whether glutamate also leads to phosphorylation of CREB at Ser142. To this end, we applied 100 μM glutamate to hypothalamic brain slices at CT 14. After 15 min of treatment, glutamate induced the phosphorylation of both CREB Ser142 and Ser133 in the SCN (Figure 2B). Thus, glutamate mimics the effect of a light stimulus, indicating that in the SCN, both CREB phosphorylations are activated due to glutamate-dependent pathways. Furthermore, the parallel patterns of CREB phosphorylation at Ser142 and Ser133 suggest a synergistic effect on CREB regulation.

Previous studies have implicated a contribution of CREB in light-induced retinal gene expression (Yoshida et al., 1998). In order to define a possible role of CREB Ser142, we compared the retinal phosphorylation levels of dark-adapted and light-stimulated animals. Whereas light strongly induced phosphorylation of CREB Ser133 in the inner nuclear and the ganglion cell layers of the retina and in the SCN, phosphorylation of CREB Ser142 could not be detected in the retina of either control or induced animals (Figure 2C). These data, therefore, suggest that in the retina, only the phosphorylation of CREB Ser133 participates in the signaling pathway of light-induced gene expression.

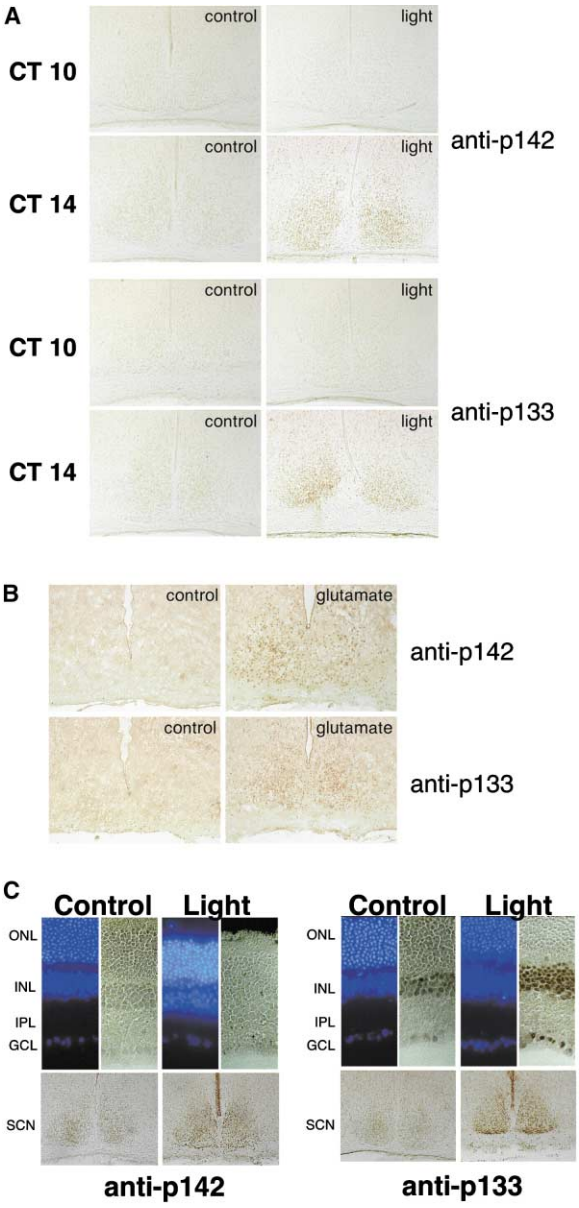


Figure 2. Light and Glutamate Induce CREB Phosphorylation at Ser142 Only in the SCN

(A) Light-induction in the SCN: C57Bl/6 mice were transferred for 48 hr into constant darkness and subjected to a light pulse (1000 lux) for 15 min at CT 10 or CT 14 and sacrificed (n = 6).

(B) Glutamate stimulation in the SCN: representative coronal cryosections of mouse hypothalamic brain slices stimulated with 100 μM glutamate at CT 14 for 15 min. Phosphorylation of CREB at Ser142 and Ser133 was revealed by immunohistochemistry.

(C) Light induction in the retina: entrained animals were transferred to constant darkness for 48 hr and were either kept in constant darkness or received a 5 min light pulse at CT 14. Responsiveness was confirmed in the same animal by determining CREB phosphorylation at Ser133 and Ser142 in the SCN. Representative Höchst-33342 staining and immunohistochemistry of retinal cryosections and hypothalamic coronal vibratome sections are shown. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; and ONL, outer nuclear layer.

Mutation of CREB at Ser142 in the Mouse Affects the Circadian Clock

To investigate the physiological importance of phosphorylation of CREB Ser142 in vivo, we generated a mouse mutant in which the CREB Ser142 was point-mutated to alanine: $CREB^{S142A}$ (Figures 3A and 3B). $CREB^{S142A}$ homozygous mutant mice lacking the CREB

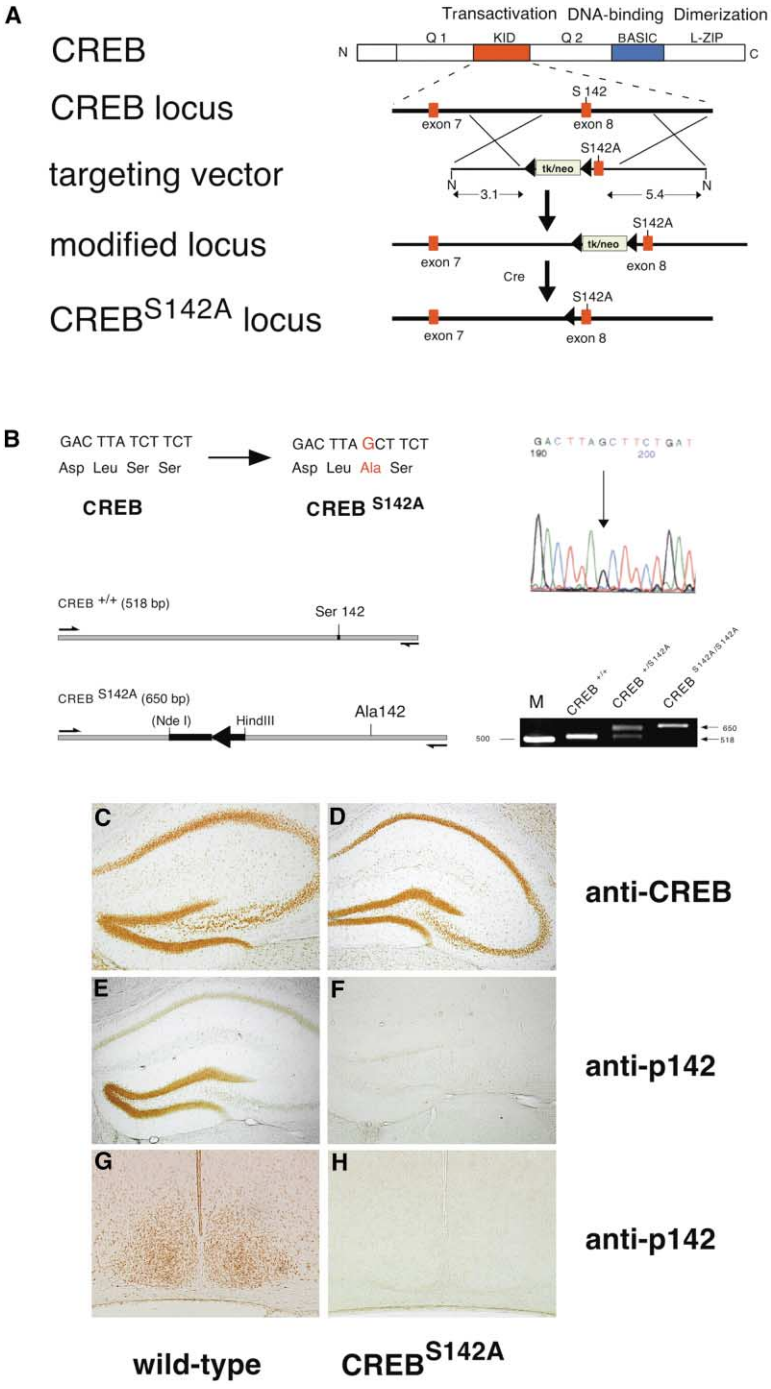
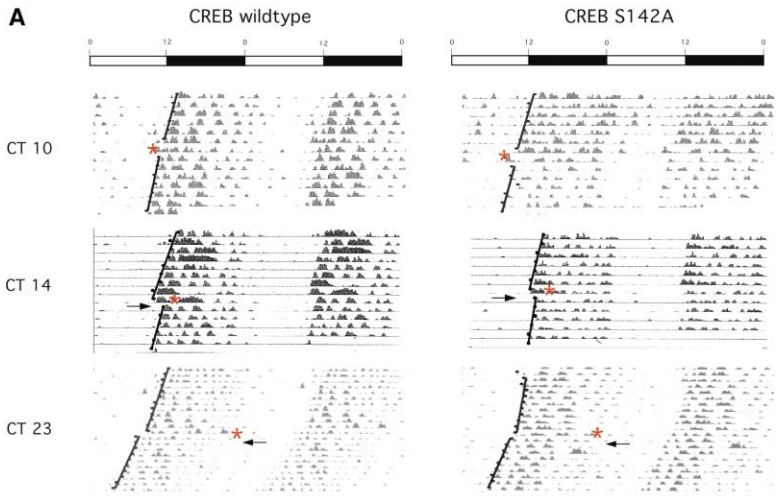


Figure 3. Generation of CREB^{S142A} Mutant Mice

(A) Targeting strategy to obtain CREB^{S142A} mice by homologous recombination in the E14/1 ES cell line using the Cre/loxP system.
(B) The presence of the CREB^{S142A} mutation was analyzed in the F1 generation by sequencing the RT-PCR product of the mutated homozygous CREB mRNA and by PCR analysis of genomic DNA. M, marker.
(C–H) In (C) and (D), CREB protein levels and phosphorylation at CREB Ser142 were determined in hippocampus (E and F) and the SCN (G and H) of wild-type and mutant animals, respectively.

Ser142 phosphorylation site are born at the expected Mendelian ratio and are fertile. Moreover, these mutants show no differences in size or morphology in comparison to wild-type littermates. The mutated CREB^{S142A} protein is expressed at similar levels as the wild-type protein (Figures 3C and 3D). As expected, phosphorylation of CREB Ser142 is absent in these animals (Figures 3E–3H). In contrast to CREB knockout mutants (Rudolph et al., 1998), the related member of the CREB family, CREM, is not upregulated (data not shown).

Since phosphorylation of CREB Ser142 is induced by light during the dark phase, we searched for an effect of the CREB^{S142A} mutation in the resetting of the central circadian clock. Homozygous CREB^{S142A} mutants and their wild-type littermates were entrained and transferred to constant darkness for at least 5 days. As expected, a light pulse at CT 14 resulted in a delayed onset of locomotor activity of -1.96 hr $\pm$ 0.21 in wild-type mice (Figure 4). However, in the CREB^{S142A} mutants, the same light pulse leads to markedly attenuated phase delays of only -1.26 hr $\pm$ 0.12 ($p = 0.00033$, $n = 8$). During late subjective night (CT 23), administration of a light pulse phase advanced the activity onset of wild-type controls by $+0.53$ hr $\pm$ 0.07. Again, the CREB^{S142A}



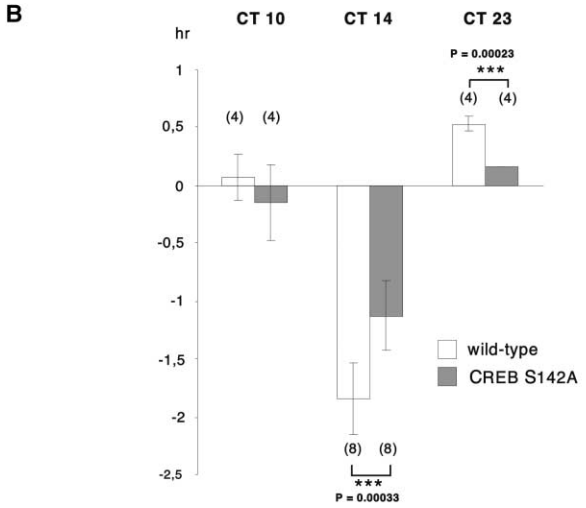


Figure 4. Impairment of Light-Induced Phase Shifts of Locomotor Activity in CREB^{S142A} Mutants

(A) Representative actograms of circadian behavior in response to a 15 min light pulse (1000 lux). Light pulses are indicated by a red asterisk.

(B) Quantification of phase shifts of circadian locomotion. Significant differences between groups were determined with a one-way ANOVA (Student's t test). Data are mean ± SEM, with the number of animals indicated in brackets. The open (0–12) and filled (12–0) bars indicate subjective day or night, respectively.

mutants displayed a reduced response: + 0.15 hr ± 0.01 ($p = 0.00023$, $n = 4$). Thus, the phosphorylation of CREB at Ser142 is involved in the molecular mechanism that synchronizes the circadian clock to the day-night cycle. Interestingly, these data indicate that CREB and the phosphorylation at Ser142 are involved in both phase advances and phase delays of circadian behavior, respectively. A control experiment at CT 10 confirmed that light treatment at CT 10 does not produce significant phase shifts of activity onset in either wild-type (0.1 hr ± 0.2) or mutant mice (−0.002 hr ± 0.1). This observation is consistent with the absence of CREB phosphorylation following light pulses at CT 10. The period length tested in light-dark or free-running conditions is comparable in wild-type and the CREB^{S142A} mutants (data not shown). Furthermore, general locomotor behavior, examined in a standard open field test at two time points over the circadian cycle, was identical in both genotypes ($n = 10$; data not shown). These results rule out a general effect of the CREB^{S142A} mutation on locomotor activity and confirm the specific involvement of CREB Ser142 phosphorylation in the resetting mechanism of the mammalian circadian clock.

We finally analyzed the molecular contribution of CREB Ser142 phosphorylation to light-induced transcription of CREB target genes in the SCN. Entrained animals were transferred to constant darkness for 48 hr and were either kept in constant darkness or received a light pulse at CT 14. At CT 15, we measured the levels of c-Fos protein by immunohistochemistry and the expression of the *mPer1* and *mPer2* genes by in situ hybridization, since they were previously shown to be strongly induced by light (Albrecht et al., 1997; Kornhauser et al., 1990; Shigeyoshi et al., 1997; Zylka et al., 1998). In the SCN of wild-type animals, the light treatment resulted in a strong induction of c-Fos protein and *mPer1* and *mPer2* mRNAs (Figures 5A and 5B). Levels of *mPer1* and *mPer2* were 4.7 and 3.7 times

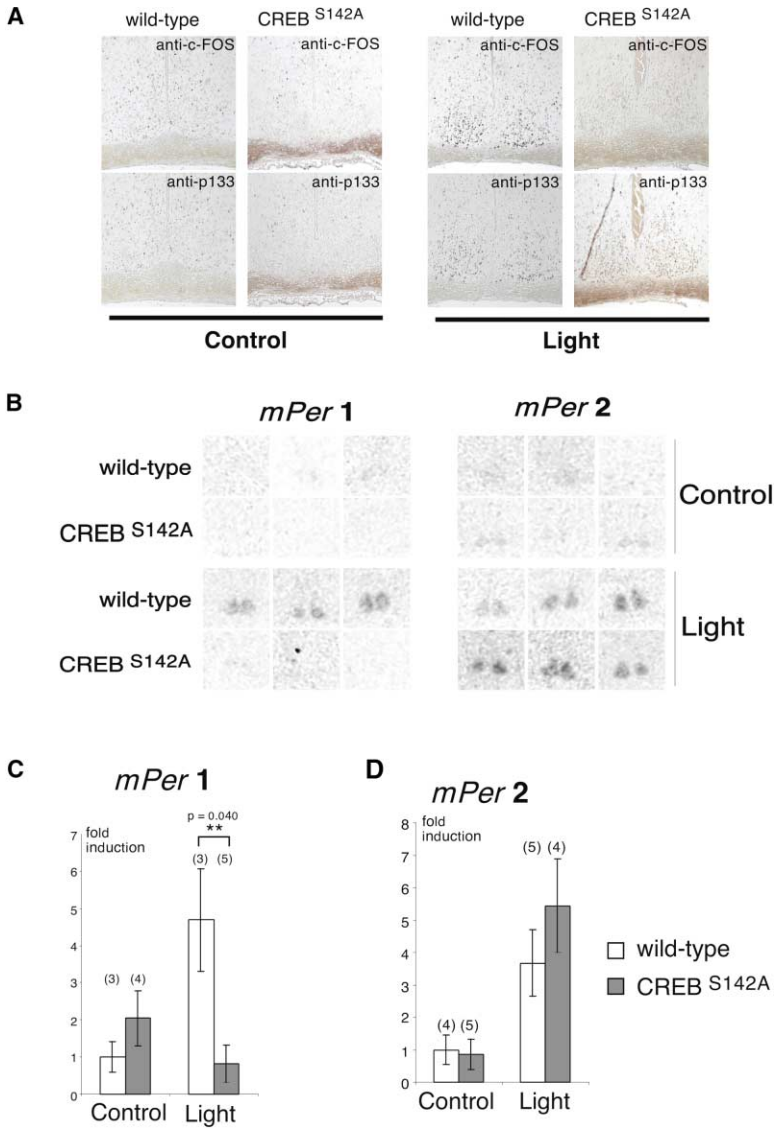


Figure 5. Light-Induced Levels of c-Fos Protein and *mPer1* mRNA Are Reduced in CREB^{S142A} Mutants

Entrained animals were transferred to constant darkness for 48 hr and either kept in constant darkness or received a 15 min light pulse at CT 14. (A) c-Fos levels in hypothalamic coronal vibratome sections. Responsiveness was confirmed in the same animal by determining CREB phosphorylation at Ser133. (B) *mPer1* and *mPer2* in situ hybridization of hypothalamic coronal cryosections and quantification (C and D). All animals were sacrificed at CT 15 (n = 3–5 per group). Significant differences between groups were determined with a one-way ANOVA (Student's t test). Data are mean ± SEM, with the number of animals indicated in parentheses.

higher than in control animals, respectively (Figures 5C and 5D). In CREB^{S142A} mutants, light-induced expression of c-Fos was significantly reduced and expression of *mPer1* was nearly abolished. In contrast, levels of phosphorylation of CREB Ser133 and *mPer2* in the SCN were comparable in wild-types and CREB^{S142A} mutants. These results demonstrate that the retinohypothalamic tract is intact in the CREB^{S142A} mutants and that the phosphorylation of CREB at Ser133 occurs independently of the phosphorylation of Ser142. Together, these findings provide genetic evidence that the phosphorylation of CREB at Ser142 contributes to the transcriptional activity of CREB.

Discussion

Synchronization of the circadian clock with the day-night cycle relies on the ability of the clock to be phase-shifted by light. An early step in this process is thought to be the phosphorylation of the transcription factor CREB at Ser133 in the suprachiasmatic nucleus (SCN). Phosphorylation-dependent activation of CREB, followed by the expression of clock genes, may lead to synchronization of the central clock to the external light-dark rhythm. Recently, it has been suggested that CREB is phosphorylated at an additional site, Ser142, probably by CaMKIIα (Sun and Maurer, 1995). In this report, we show that this phosphorylation occurs in specific brain regions, including the SCN. In the SCN, light pulses or glutamate treatment during early subjective night (CT 14) result in rapid phosphorylation of CREB Ser142, implying a role in the resetting of the circadian clock. This role was confirmed by disruption of the CREB Ser142 phosphorylation site by point mutation in the mouse (CREB^{S142A}). Light-induced expression of c-Fos, *mPer1* in the SCN, and phase shifts of the circadian locomotor activity are strongly attenuated in these mutants.

In order to understand the molecular pathway leading from perception of the light stimulus to phase shift of the central clock and, finally, to advanced or delayed circadian behaviors, several studies have been performed: inhibition of the CaMK pathways by KN-93 (Fukushima et al., 1997; Golombek and Ralph, 1994; Yokota

et al., 2001), hypothalamic injections of *mPer* antisense oligonucleotides (Akiyama et al., 1999; Wakamatsu et al., 2001), and disruption of the c-*fos* gene (Honrado et al., 1996). Strikingly, in all cases, phase delays in response to light are attenuated as is observed in the CREB^{S142A} mutant. Interestingly, CaMKIIα is thus far the only enzyme known to phosphorylate CREB at Ser142. It is, therefore, tempting to speculate that CaMKIIα activity, CREB phosphorylation at Ser142 and Ser133, and *mPer1* expression contribute to a common signaling cascade during the resetting of the circadian clock: glutamate released in the neurons of the SCN activates several pathways, such as the MAPK and the CaMKIIα pathway leading to the phosphorylation of CREB at Ser133 and Ser142. Whereas CREB function is generally regulated by phosphorylation at Ser133, in the SCN, the convergence of an additional pathway leading to the phosphorylation of Ser142 is required for specific expression of CREB target genes. Phosphorylation of CREB at Ser142 has been shown in vitro to disrupt the binding of CREB to the KIX domain of CBP (Parker et al., 1998; Kornhauser et al., 2002). Together with our in vivo data, this observation would suggest that the transcriptional activation of CREB, when phosphorylated at Ser142, may be mediated by a KIX-independent mechanism involving either a different domain of CBP or, alternatively, the recruitment of a different coactivator. The parallel phosphorylations of CREB Ser142 and Ser133 in the SCN, the impaired expression of c-Fos and *mPer1*, and the attenuated phase shifts in the CREB^{S142A} mutants suggest that CREB requires phosphorylation of both sites in order to induce maximal phase shifts. These in vivo findings differ from previous in vitro experiments, where CREB Ser142 phosphorylation of CREB-Gal4 fusions seemed to inhibit transcription of a luciferase-reporter in transient transfections (Sun et al., 1994).

In summary, our data provide evidence that CREB is phosphorylated in vivo at Ser142, indicating that several signaling pathways may contribute to the regulation of CREB transcription by a combinatorial phosphorylation code. In the suprachiasmatic nucleus, light pulses or glutamate treatment at CT 14 result in rapid phosphorylation of CREB Ser142. Disruption of this phosphorylation site in the mouse strongly attenuates light-induced expression of c-Fos, *mPer1*, and phase shifts of the circadian locomotor activity. These results genetically confirm a function of mammalian CREB in the central circadian clock and implicate phosphorylation of Ser142 in the transcriptional regulation of CREB. Finally, they support a role of CREB Ser142 phosphorylation in the light-induced resetting mechanism of the mammalian clock.

Experimental Procedures

Phosphospecific Antibody

Polyclonal antibodies against CREB phosphorylated at Ser142 (anti-p142) were generated by immunization of rabbits with the phosphorylated peptide (K-I-L-N-D-L-S-phospho-S-D-A-P-G-V-P-R-C-COOH; aa 136–151). The serum (Eurogentec, Seraing, Belgium) was precipitated with ammonium sulfate, affinity purified with immobilized phosphopeptide using the SulfoLink Kit (Pierce, IL). The anti-p142 was used at 1:10000 for Western blot analysis and at 1:1000 in immunohistochemistry. For peptide competitions, the anti-p142 was preincubated overnight with 1 mg/ml of the phosphorylated or unphosphorylated form of the peptide used for immunization and then applied to vibratome sections.

Immunoblotting

Brains were immediately frozen in liquid nitrogen and stored at −80°C. Nuclear cell extracts were prepared from tissue homogenized in 0.4 M RIPA (1 mM sodium vanadate, Calyculin A), as described in Herdegen et al., 1994. GST-CREB was expressed in BL21/pLys bacteria and purified on a glutathione sepharose 4B (Amersham Pharmacia Biotech). Samples were resolved by SDS-PAGE and proteins were detected by immunoblotting.

Immunohistochemistry

Mice were sacrificed by decapitation, and brains were fixed by immersion into cold 4% paraformaldehyde at 4°C overnight. Brains were cut at a thickness of 50 μm on a vibratome (Leica) ,and floating sections were further processed for immunohistochemical detection using the VECTASTAIN ABC system (Vector Laboratories). Quantitative analysis of the signal was performed using a Zeiss axiophot microscope and the IBAS image processing system, as previously described (Bock et al., 1991). Imaging of the oscillating phosphorylations was performed by linear regression using the S-Plus 3.4 Software-Package (MathSoft, Inc., Seattle). Antibodies used were: CREB (1:9000; Herdegen et al., 1994), CREB phospho-Ser133 (1:5000; Herdegen et al., 1994 or 1:500; New England Biolabs), and c-Fos (1:3000; Oncogene Ab-5).

Cloning Strategy for the Generation of the CREB^{S142A} Mutants

A 2.5 kb fragment of the murine CREB gene, which included exon 8, was subcloned from a λ phage library. The point mutation of CREB Ser142 to Ala142 was introduced by overlap PCR (P1: 5′-AATGACTTAG CTTCTGATGC ACCAGGG, P2: 3′-CCCTGGTGCA TCAGAAGCTA AGTCATT, P3: 5′-AAGACCCAG ATTCAATCCC, and P4: 3′-GGGGCTGAAG TCTCCTCTTC). In the same fragment, a loxP-thymidine-kinase/neomycine phosphotransferase-loxP selection cassette (Reichardt et al., 1998) was introduced 192 bp upstream of exon 8 into a NdeI site. The final targeting vector contained 12.4 kb. Electroporation of the targeting construct, removal of the selection cassette, and selection of positive clones was performed as described (Reichardt et al., 1998). One clone was for blastocyst injections to produce six male chimeric animals, which were crossed to C57Bl/6. Sequencing of the mutated allele was performed by RT-PCR using the following conditions: 94°C, 1 min; 52°C, 1 min; 72°C, 1 min; 35 cycles: (5′ primer: TGGAGTGAGT AAGGGGAGAT and 3′ primer: TGACAGTAGG GCAAACAATA). For genotyping, we used the following PCR conditions: 94°C, 1 min; 52°C, 1 min; 72°C, 1 min; 35 cycles (5′ primer: AAGACCCAG ATTCAATCCC and 3′ primer: GGGGCTGAAG TCTCCTCTTC). The expected fragment sizes were 621 bp for the wild-type allele and 753 bp for the homozygous mutant allele, respectively. For circadian behavior study, we backcrossed the animals at least three generations into C57Bl/6 prior to analysis.

Hypothalamic Slice Culture

Mice kept under standard 12 hr light:12 hr dark conditions were decapitated, and brains were quickly removed. 400 μm coronal hypothalamic slices were cut at 4°C on a vibratome. To avoid phase shifting of the circadian clock by brain slicing, we prepared slices before CT 10, that is, at least 2 hr before the onset of the dark phase. Stimulations of the slices were performed subsequently at CT 14. After preincubation for at least 2 hr in artificial CSF (aCSF) (145 mM NaCl; 5 mM KCl; 1.8 mM $CaCl_2$; 0.8 mM $MgCl_2$; 10 mM HEPES; and 10 mM glucose [pH 7.35] at 37°C), slices were stimulated with 100 μM glutamate for 15 min. Unstimulated slices served as controls. Subsequently, slices were fixed with 4% PFA for 12–16 hr, cryoprotected (20% sucrose in PBS), and sectioned on a cryostat. 14 μm sections were mounted on gelatin-coated slides and stored at −20°C until immunocytochemistry was performed.

In Situ Hybridization

Immediately after removal, brains were frozen in isopentane (4 min at 20°C) and stored at −70°C until use. Serial coronal brain cryosections of 10 μm were prepared using standard procedures. In situ

hybridizations with sections of the SCN were performed as described previously (Damiola et al., 2000). Levels of *mPer1* and *mPer2* were quantified using the Quantify One (BioRad Laboratories) system.

Animal Experiments

Single caged adult C57Bl/6 mice or CREB^{S142A} mutants and their wild-type littermates were entrained during at least 7 days to a 12 hr light:12 hr dark cycle. The animals were then transferred to constant darkness and subjected to a light pulse of 1000 lux for 15 min. The circadian behavior was analyzed on adult homozygous CREB^{S142A} and wild-type littermates and viewed using the ClockLab software package (Actimetrics, Evanston, IL) developed in MatLab (Mathworks, Inc., Seattle), as described in von Gall et al. (1998). Phase shifts were quantified as the time difference between regression lines of activity onsets before and after light application.

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